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Rapid and Sensitive Detection of Noroviruses by Using TaqMan-Based One-Step Reverse Transcription-PCR Assays and Application to Naturally Contaminated Shellfish Samples

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Noroviruses (NoV), which are members of the family *Caliciviridae*, are the most important cause of outbreaks of acute gastroenteritis worldwide and are commonly found in shellfish grown in polluted waters. In the present study, we developed broadly reactive one-step TaqMan reverse transcription (RT)-PCR assays for the detection of genogroup I (GI) and GII NoV in fecal samples, as well as shellfish samples. The specificity and sensitivity of all steps of the assays were systematically evaluated, and in the final format, the monoplex assays were validated by using RNA extracted from a panel of 84 stool specimens, which included NoV strains representing 19 different genotypes (7 GI, 11 GII, and 1 GIV strains). The assays were further validated with 38 shellfish cDNA extracts previously tested by nested PCR. Comparison with a recently described real-time assay showed that our assay had significantly higher sensitivity and was at least as sensitive as the nested PCR. For stool specimens, a one-step duplex TaqMan RT-PCR assay performed as well as individual genogroup-specific monoplex assays. All other enteric viruses examined were negative, and no cross-reaction between genogroups was observed. These TaqMan RT-PCR assays provide rapid (less than 90 min), sensitive, and reliable detection of NoV and should prove to be useful for routine monitoring of both clinical and shellfish samples.

Noroviruses (NoV) have emerged as a leading cause of outbreaks of acute viral gastroenteritis worldwide and have been identified as the primary pathogens associated with shellfish-borne gastroenteritis (16, 19, 32). Since NoV is mainly transmitted through the fecal-oral route and the infectious dose has been reported to be low (24), the risk of infection after consumption of raw or improperly cooked seafood or after exposure to contaminated recreational water is considered high (19). Genetically, NoV can be subdivided into five separate genogroups (genogroup I [GI], GII, GIII, GIV, and GV), and viruses belonging to GI, GII, and GIV have been detected in humans (17). Within the human genogroups, NoV strains can be further delineated into at least 31 genetic clusters or genotypes based on the capsid N-terminal/shell domain (13). Although reverse transcription-PCR (RT-PCR) has become the standard for diagnosis of NoV infection worldwide (1, 5, 36), many laboratories use multiple sets of primers targeting different regions of the genome because of the difficulty of designing broadly reactive primers that are able to sensitively detect all the different NoV genotypes (36). In addition, confirmatory steps are needed to evaluate the specificity of the amplification product (5, 34, 35) or to increase the sensitivity for detection of NoV in food and water matrices (6, 18). Recently, several SYBR Green and TaqMan-based RT-PCR assays for the detection of NoV have been reported (2, 10, 12, 23, 28). However, none of these assays used a one-tube or duplex format or were validated with naturally contaminated environmental (shellfish) samples. Most of these TaqMan-based assays targeted a region just downstream of region B (5) near the ORF1-ORF2 junction, which had been shown to be the most conserved region of the NoV genome (14). Crucial for the sensitive detection of NoV in environmental samples like shellfish is a rapid assay with broad reactivity as well as a low detection limit. Real-time PCR assays show the greatest promise due to their simplicity and ability to generate quantitative data. In this paper, we report the development of a one-step TaqMan RT-PCR assay for the sensitive detection of GI and GII NoV strains and its validation with panels of archived stool specimens and naturally contaminated shellfish samples.

MATERIALS AND METHODS

Clinical strains and shellfish samples. A panel of 84 stool specimens collected over a 5-year period (1996 to 2000) was derived from both outbreaks and sporadic cases of gastroenteritis. These specimens were tested previously for NoV by RT-PCR by using primers targeting conserved regions of ORF1 that include the GLPSG motif of the RNA-dependent RNA polymerase (11, 34) or region B (5). The specimens had been stored at −80°C and represented most of the NoV genotypes that had recently been circulating in Europe and the United States (Table 1). In addition, eight specimens containing other enteric viruses (adenovirus type 40 and type 41, echovirus 11, rotavirus strain SA-11, hepatitis A virus strain HM175, and poliovirus Sabin type 1) were included in this study. Stool samples were diluted to obtain 10% preparations in phosphate-buffered saline (PBS), vortexed, and clarified by centrifugation at 2,000 × *g* for 15 min.

All shellfish used in this study were collected from commercial harvesting areas in the north Atlantic region during 2002 to 2004. Altogether, 36 samples of Pacific oysters (*Crassostrea gigas*) and two samples of native oysters (*Ostrea edulis*) were used. For each sample a minimum of six shellfish were aseptically opened, and the animals were removed from their shells. The peripheral flesh and organs of each animal were then cut away from the hepatopancreas, which was then finely chopped as described previously (9). To degrade the shellfish

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TABLE 1. Norovirus-positive stool samples used in this study

Genotype	Prototype[a]	Strain(s)
GI.1	Norwalk	8FIIb
GI.2	Southampton	c2, S7, sw8
GI.3	Desert Shield	c3, S10, sw6, sw18
GI.4	Chiba	B39, c4, S11, sw1, sw2
GI.5	Musgrove	c5
GI.6	Hesse	B18, B49, sw3
GI.7	Winchester	c7
GII.1	Hawaii	sw7, R4, R18, sw16, c8, G4
GII.2	Melksham	SMV1-4, S2, R10, B13, B16, c9, sw5
GII.3	Toronto	B2, B3, B40, G4, c10, S13, R8, R9, R21, J8b, J21a, J21b, J24, sw10, sw13, sw14
GII.4	Bristol	B4, B26, B27, B28, B45, B48, B51, S19, sw17, sw19, sw20, sw22, sw23, sw24, G1, G2, c11
GII.5	Hillingdon	c12
GII.6	Seacroft	c13, G3, R16, B11, J11, R1, sw11, sw12
GII.7	Leeds	c14, J9a, M13
GII.8	Amsterdam	B5
GII.9	Virginia	J10b
GII.12	Wortley	M17, J1a, J15a
GII.14	Fayetteville	M12, M27
GIV.1	Alphatron	c17

[a] Data from reference 7 and S. Monroe (unpublished data).

tissue and allow the release of virions into solution, an equal volume of a 100-μg/ml proteinase K solution was added, and the sample was then incubated at 37°C for 1 h with shaking at 320 rpm. The reaction mixture was subsequently incubated at 65°C for 15 min in order to inactivate the proteinase, and the soluble portion (shellfish concentrate) was collected by centrifugation at 3,000 × *g* for 5 min and stored at 4°C.

Viral RNA extraction. For stool samples, viral RNA was extracted from the clarified PBS extracts by using QIAamp microspin columns (viral RNA mini kit; QIAGEN, Valencia, Calif.) according to the manufacturer's instructions and was stored at −80°C. For each shellfish sample, one 400-μl aliquot and one 133-μl aliquot of shellfish homogenate for nested PCR analysis and one to three 133-μl aliquots of shellfish homogenate for real-time PCR analysis were added to separate 1.5-ml microcentrifuge tubes containing 10 μl of a silica bead suspension (glassmilk: Anachem Ltd., Luton, United Kingdom). The final volumes were adjusted to 410 μl by adding PBS (Oxoid, Basingstoke, United Kingdom). Viral RNA from purified shellfish concentrates was extracted by binding to size-fractionated silica after treatment with guanidine isothiocyanate as described previously (4, 20). After elution from the silica particles, the RNA was precipitated in ethanol and sodium acetate. Each RNA pellet was resuspended in 8.9 μl of RT reaction mixture and immediately subjected to reverse transcription as described previously (6)

Reverse transcription and nested PCR for shellfish. cDNA was generated by using random hexamers, and the first round of PCR was performed by using the G1-G2 and SM31 primers (6); this was followed by second-round amplification by using 1 μl of the first-round amplicon and 49 μl of a PCR mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.25 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1 U of AmpliTaq Gold enzyme (ABI), and 20 pmol of each primer (Ando and E3 for GI and Ni and E3 for GII). The reaction mixtures were then analyzed by agarose gel electrophoresis. RT-PCR products of the expected size (the GI and GII primers amplified 116- and 114-bp products, respectively) were cloned and sequenced by using previously described methods (8). Phylogenetic analysis was performed by using the Lasergene software (DNAStar). An NoV sequence in either of the two reactions for each shellfish sample was counted as an overall positive result for that sample.

TaqMan RT-PCR primer and probe design. To identify conserved regions in the NoV genome that are suitable for the development of real-time RT-PCR primers and probes, NoV sequences were obtained from databases available at the University of North Carolina at Chapel Hill, CEFAS, and GenBank. Several regions in ORF1 and ORF2 were considered, and based on multiple alignments several candidate primers and probes were selected for evaluation, including recently published TaqMan reagents (12). Although initially several promising primer sets targeting region C (15, 27) and region D (33) were identified and evaluated, we selected genogroup-specific primers and probes targeting the conserved region at the ORF1-ORF2 junction of the genome (Table 2). We used the Metcalf software (30) to design consensus primer and probe sequences by calculating the melting temperature of the primer with each individual NoV sequence used in this study. Care also was taken to avoid any primer-primer self-annealing. Primer JJV1F included a single deoxyinosine at position 12 to compensate for a fourfold degeneracy. In the final format, the GI primers (JJV1F and JJV1R) amplified a 96-bp fragment. A TaqMan probe (JJV1P) was designed to detect all GI strains (Tables 1 and 2). The GII primers amplified an 89-bp fragment, and the forward primer (JJV2F) was designed to hybridize to all genetic clusters of genogroup II. 6-Carboxyfluorescein (FAM)-5′-labeled probes were used for monoplex assays, whereas in duplex assays a FAM-labeled GI probe and a 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein (JOE)- or 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (TET)-labeled GII probe were used. The probes contained either the 3′ Black Hole quencher dye (biotechnology core facility at Centers for Disease Control and Prevention, Atlanta, Ga.) or TAMRA (6-carboxytetramethylrhodamine; Applied Biosystems, Foster City, Calif.) for the analyses of shellfish.

TaqMan RT-PCR assays. For two-step TaqMan assays, random hexamers (ABI) were compared to specific reverse primers by using a GeneAmp RNA PCR core kit (Applied Biosystems). RT was performed at 42°C for 15 min, followed by incubation at 99°C for 5 min and cooling to 5°C for 5 min in a GeneAmp 9700 PCR system (Applied Biosystems). Then TaqMan PCR was performed with a QuantiTect probe PCR kit (QIAGEN).

In the final format, the reaction mixtures contained 0.5 or 1 μl of template RNA (stool samples) or 2 μl of cDNA (shellfish samples), each primer at a concentration of 250 nM, 100 nM probe JJV1P (for GI viruses) or 100 nM probe RING2-TP (for GII viruses) (12), and QuantiTect probe RT-PCR buffer (QIAGEN) for stool samples or TaqMan universal PCR master mixture (Applied Biosystems) for shellfish samples. Duplex protocols included primers (200 nM), GI probe (100 nM), and GII probe (50 nM). For stool samples, the reaction mixture was then subjected to a one-step assay by using the following amplification conditions: (i) RT for 30 min at 55°C, (ii) 15 min at 95°C to activate *Taq* polymerase, and (iii) 45 cycles of 10 s at 94°C, 20 s at 55°C, and 15 s at 72°C. cDNA from shellfish samples was amplified as follows: 2 min at 50°C and then 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Several thermocyclers were used to perform the TaqMan RT-PCR assay developed in this study; these thermocyclers included an iCycler iQ (Bio-Rad, Hercules, Calif.), an R.A.P.I.D. Cycler (Idaho Technology Inc., Salt Lake City, Utah), and a SmartCycler (Cepheid, Sunnyvale, Calif.) for clinical samples and an ABI

TABLE 2. Oligonucleotides for TaqMan-based NoV RT-PCR used in this study

Genogroup	Oligonucleotide	Sequence (5′-3′)[b]	Orientation	Location[c]
GI	JJV1F	GCC ATG TTC CGI TGG ATG	+	5282–5299
	JJV1R	TCC TTA GAC GCC ATC ATC AT	−	5377–5358
	JJV1P	FAM-TGT GGA CAG GAG ATC GCA ATC TC-BHQ	+	5319–5341
GII	JJV2F	CAA GAG TCA ATG TTT AGG TGG ATG AG	+	5003–5028
	COG2R[a]	TCG ACG CCA TCT TCA TTC ACA	−	5100–5080
	RING2-TP[a]	FAM-TGG GAG GGC GAT CGC AAT CT-BHQ	+	5048–5067

[a] See reference 12.
[b] In duplex assays a 5′-FAM-labeled GI probe was combined with a 5′-JOE- or 5′-TET-labeled GII probe. BHQ, Black Hole quencher.
[c] Nucleotide positions based on the Norwalk (GI) (accession no. M87661) and Lordsdale (GII) (accession no. X86557) sequences.

GeneAmp SDS 5700 real-time PCR machine (Applied Biosystems) for shellfish samples.

All amplification reactions were carried out in duplicate. To determine the correct product size and to compare the sensitivity with the sensitivity of fluorescence-tagged detection, RT-PCR products of TaqMan reactions from clinical samples were visualized on ethidium bromide-stained agarose gels. Shellfish samples that gave a positive result in either or both of the duplicate reactions were counted as overall positive samples.

Standards for real-time RT-PCR assay. To obtain representative positive control standards, amplicons generated from strains S11 (GI genotype 4 [GI.4]) and SMV1-4 (GII.2) were cloned using a TrueBlue MicroCartridge PCR cloning kit (Genomics One, Quebec, Canada), and plasmid DNA was purified by using a Nucleobond 100 kit (Promega). The concentration of each purified plasmid was determined by spectroscopy at 260 nm, and 10-fold serial dilutions of each plasmid DNA were used for preparation of the standard curve.

Discrepancy testing. Discrepancy testing was performed when any shellfish cDNA sample resulted in a discrepant TaqMan result compared to the corresponding nested PCR result for the same genogroup. Duplicate 2-μl aliquots of the cDNA used for the TaqMan analysis were analyzed by nested PCR (6). Samples that gave a single positive duplicate reaction were counted as overall positive samples, and the result superseded the original nested PCR result if it was different, for the purposes of comparative analysis.

RESULTS

Specificity of genogroup-specific TaqMan RT-PCR assays. For detection of NoV in clinical samples, we used a one-step RT-PCR approach. For shellfish extracts, random primer-generated cDNA was used, and hence a two-step approach was used. The individual GI and GII TaqMan RT-PCR assays were validated by using a panel of 84 stool specimens containing NoV RNA, as detected by conventional RT-PCR (33, 34). All RT-PCR products had the appropriate size when analyzed on ethidium bromide-stained agarose gels (data not shown). The GI and GII TaqMan assays were genogroup specific, and no cross-reactions with strains from other genogroups were observed. Based on these results, we designed a one-step GI-GII duplex assay for sensitive detection and for distinguishing the most prevalent genogroups. In the optimized duplex format, we used a 5′-FAM-labeled GI probe and a 5′-JOE-labeled GII probe. GIV-specific primers and a TaqMan probe were also developed, but since only one GIV-positive stool sample was available for analysis, this assay was not validated further.

Limit of detection of NoV TaqMan assays. The sensitivity of each monoplex assay was determined using 10-fold serial dilutions of a NoV GI or GII plasmid (Fig. 1). To determine the sensitivity of the real-time RT-PCR assays, standard curves of crossing point (Cp) values versus the number of copies were generated by analyzing four replicates of 10-fold serial dilutions of the NoV GI plasmid (7.2×10^6 to 7.2×10^0 copies) and of the NoV GII plasmid (5.9×10^6 to 5.9×10^0 GII.2 copies). In all cases, an excellent correlation between the amount of target template and the Cp value was obtained (Fig. 1), and the sensitivity of both NoV TaqMan RT-PCR assays was calculated to be <10 copies of viral genome per reaction.

Parameters of NoV TaqMan RT-PCR assays. Several different variables of the assay (random priming versus specific primer for RT, two-step format versus one-step format, and monoplex versus duplex [GI and GII combined]) were compared. By using a panel of eight strains (four GI strains and four GII strains), we determined that the Cp values were lower (range, 2.2 to 7.8) when specific primers were used than when random primers were used, whereas the differences between the two-step format and the one-step format were less than a Cp value of 1 (data not shown). A total of 25 NoV clinical samples representing strains of all genotypes available in this study were analyzed by duplex and monoplex assays. The duplex assay (GI FAM labeled and GII JOE labeled) had a sensitivity comparable to that of the monoplex assay (FAM-labeled probe), and the average Cp values of the duplex assay were less than 1.0 higher than the average Cp values of the monoplex assay (data not shown).

Comparison of NoV TaqMan assays with recently described NoV TaqMan assays. To compare the sensitivity of our monoplex NoV TaqMan assays with the sensitivity of the assay recently described by Kageyama et al. (12), we analyzed a panel of 17 representative strains using a one-step RT-PCR format. Overall, our real-time assay was 10- to 100-fold more sensitive (with Cp values that were 4.12 ± 2.16 lower) (Table 3). In parallel, a small panel of samples was also analyzed by another NoV TaqMan assay (10), but a full comparison was not performed because this assay did not detect any of the GI strains (data not shown). The only difference between our GII assay and the assay described by Kageyama et al. is the sequence of the forward primer (which Kageyama et al. designed with several degeneracies, but which we designed with no degeneracies). To evaluate the robustness of our assay versus the robustness of the assay of Kageyama et al. (12), we compared the two assays by using gradient real-time RT-PCR. The profiles for Cp value versus temperature for a panel of five different GII strains clearly showed that primer JJV2F yielded consistent low Cp values across the entire range of annealing temperatures tested (54 to 62°C), whereas the COG2F primer yielded increasingly higher Cp values as the annealing temperature increased and no fluorescence signal at 60°C or higher (Fig. 2).

Detection of NoV in shellfish extracts. Eighteen of the 38 shellfish extracts tested positive for GI NoV by both nested PCR and TaqMan RT-PCR (Table 4). Altogether, six extracts showed discordant results; four samples were positive only by TaqMan RT-PCR, and two samples were positive only by nested PCR. Of the 38 extracts, 19 were positive for GII by both nested PCR and TaqMan RT-PCR. Five TaqMan RT-PCR-positive samples could not be confirmed by nested PCR, and one sample was positive by nested PCR and negative by TaqMan RT-PCR (Table 4). Nested PCR products were sequenced and genotyped and will be published elsewhere (J. Lowther, J. Vinjé, N. Jothikumar, K. Henshilwood, and D. Lees, submitted for publication).

The Cp values for shellfish samples were at or near the limit of sensitivity of the assay, and frequently only one of two replicates was positive. With the GI assay, 55% of the positive samples were positive for only one replicate, with Cp values ranging from 39 to 43. However, for the samples for which both replicates were positive, the majority (80%) had Cp values of <40. With the GII assay, the Cp values ranged from 38 to 46. Compared to the GI real-time assay, fewer samples were positive for only one duplicate (25% of positive samples), but a lower proportion of samples had average Cp values of <40.

DISCUSSION

NoV are the most common cause of food-borne disease, and fecally contaminated shellfish are some of the most important

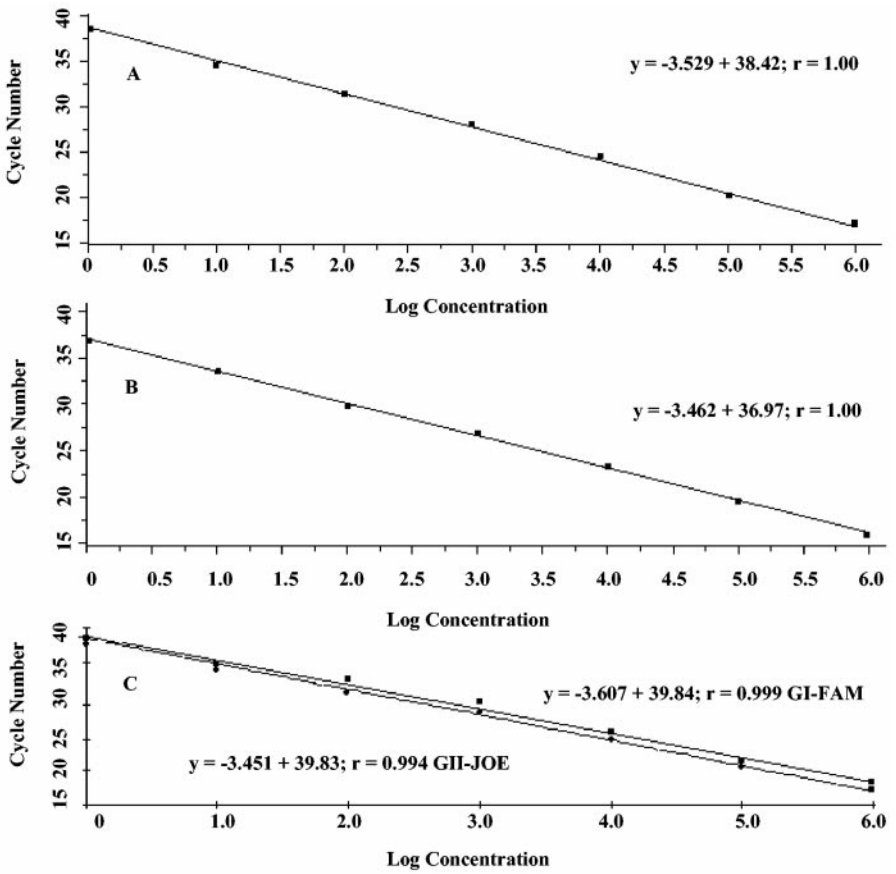

FIG. 1. Standard curves for GI.4 and GII.4 plasmid standards containing from 1 to 10^7 copies per reaction. (A) Detection limit for the NoV GI TaqMan assay based on a dilution series of the GI.4 plasmid with the R.A.P.I.D. Cycler. (B) Detection limit for the NoV GII TaqMan assay based on a dilution series of the GII.4 plasmid with the R.A.P.I.D. Cycler. (C) Detection limit for the NoV duplex TaqMan assay based on a dilution series of the GI.4 and GII.4 plasmids with the Bio-Rad iCycler iQ.

food items involved in such outbreaks (17, 29, 32). Hence, introduction of routine norovirus monitoring of shellfish would be highly advisable, but most reported conventional RT-PCR assays are unable to detect all human noroviruses (36). Although nested PCR assays have been successfully employed for amplification of low levels of virus found in naturally contaminated shellfish (6, 21, 22, 25, 26, 31), there is currently no international approved standardized method, and to our knowledge, no laboratory offers an accredited service for detection of NoV in shellfish.

In the present study we designed and evaluated broadly reactive one-step TaqMan-based RT-PCR assays for the sensitive detection of genogroup I and II NoV. The assays were validated by using the full range of NoV genotypes from clinical samples and were shown to be at least as sensitive and broadly reactive as conventional nested PCR for detection of noroviruses in shellfish samples. Initially, several promising generic primer sets targeting region C (15, 27) and region D (33) of GI and GII NoV were identified and evaluated for real-time RT-PCR (data not shown), but in the final protocol, the ORF1-ORF2 junction region was selected because this region has been shown to have the highest sequence similarity across the genome (12).

To balance the maximum number of mismatches between

TABLE 3. Comparison of Cp values of UNC NoV and Kageyama NoV TaqMan RT-PCR assays by using RNA from 20 NoV strains[a]

Strain (genotype)	Cp values		Difference
	NoV TaqMan RT-PCR assay		
	This study	Study of Kageyama et al.[b]	
Sw8 (GI.2)	23.7	28.2	4.5
Sw6 (GI.3)	23.8	26.0	2.2
Sw18 (GI.3)	24.2	32.3	8.1
Sw1 (GI.4)	23.8	30.4	6.6
Sw3 (GI.6)	28.8	36.1	7.3
B18 (GI.6)	29.2	38.1	8.9
Sw17 (GII.1)	18.6	23.8	5.1
CDC8 (GII.1)	24.8	27.6	2.8
CDC9 (GII.2)	23.5	27.6	4.1
R10 (GII.2)	29.0	34.1	4.9
S13 (GII.3)	26.6	35.1	8.5
B40 (GII.3)	22.9	29.1	6.2
R9 (GII.3)	25.0	29.1	4.1
B27 (GII.4)	23.9	25.9	2.0
Sw10 (GII.4)	29.9	33.9	4.0
B4 (GII.4)	27.6	30.0	2.4
CDC12 (GII.5)	29.3	33.9	4.6
CDC13 (GII.6)	35.1	37.2	2.1
R16 (GII.6)	21.2	22.8	1.6
CDC14 (GII.7)	28.3	33.8	5.5

[a] UNC, University of North Carolina at Chapel Hill.
[b] See reference 12.

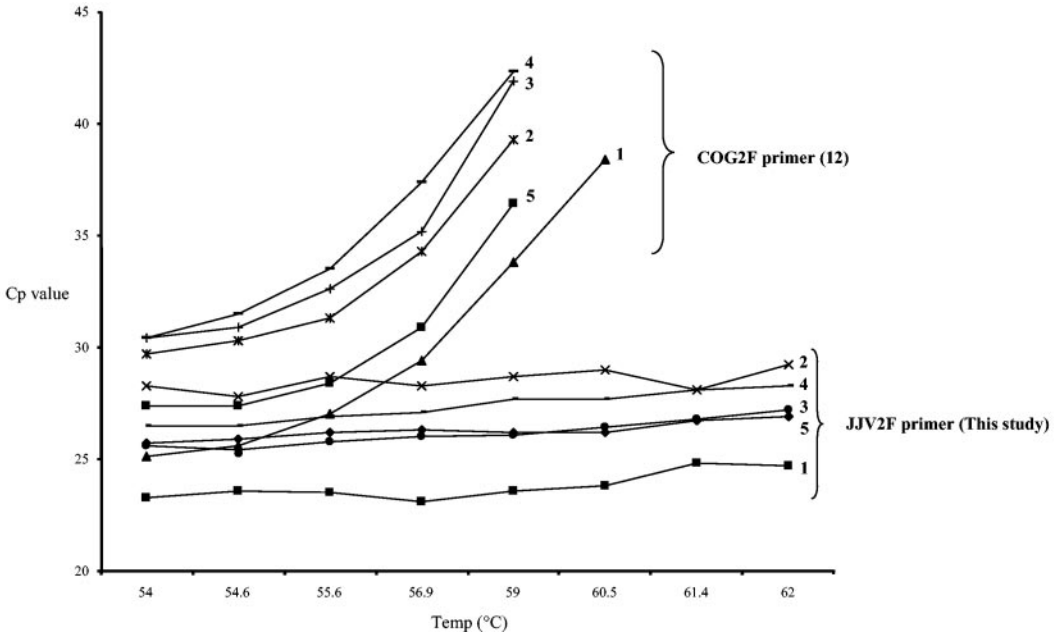

FIG. 2. Comparison of Cp values for two different GII forward primers, JJV2F (this study) and COG2F (12), determined by gradient TaqMan RT-PCR with RNA from five different GII strains (lines 1, R4 [GII.1]; lines 2, c8 [GII.1]; lines 3, c9 [GII.2]; lines 4, B2 [GII3]; lines 5, sw12 [GII.6]).

primer and template, the GII forward primer JJV2F was designed in such a way that it contained intentionally introduced ambiguities. This was accomplished by calculating the melting temperaure for the interaction of both primers with each individual genotype. Primer JJV2F exhibits at least 70% homology with all of the different GII genotype target sequences, while the eight bases at the 3′ end of the primer are 100% complementary to most known GII genomic sequences. Because of the mismatches in the forward primer, we chose a relatively low annealing temperature (55°C) rather than 60°C to guarantee binding to all NoV GII targets. At this lower annealing temperature, we did not observe any nonspecific amplification when analyzing the TaqMan RT-PCR products on agarose gels (data not shown). The success of this approach was demonstrated when we compared our novel assays with recently described NoV TaqMan assays (10, 12). The gradient real-time RT-PCR data demonstrated the increased robustness of our GII assay with the forward primer without degeneracy, further supporting the importance of using nondegenerate primers when optimal sensitivity is required. In addition, our data show that a one-step duplex format is able to detect GI NoV or GII NoV in clinical samples without substantial loss of sensitivity compared to the monoplex format. However, because the level of NoV contamination in shellfish is close to the limit of detection, the performance of the duplex assay on shellfish templates needs to be evaluated further. Our monoplex assays were able to detect <10 copies of viral genome per reaction. However, because the sensitivity was calculated based on plasmid DNA, which does not factor in the role of reverse transcriptase, the use of RNA run-off transcripts should give a more accurate estimate.

TABLE 4. Comparison of the performance of TaqMan RT-PCR assays and the performance of nested PCR for the detection of norovirus RNA in 38 shellfish extracts

Genogroup	Nested PCR result	TaqMan RT-PCR	
		No. of samples positive	No. of samples negative
I	Positive	18	2
	Negative	4	14
II	Positive	19	1
	Negative	5	13

To date, only two studies in which TaqMan technology has been used for the detection of NoV in naturally contaminated shellfish samples have been reported (23, 26), and both used degenerate primers and probes that were described by Kageyama et al. (12). The increased sensitivity of our assay in terms of Cp values compared with other real-time RT-PCR assays is likely due to the use of GII primers and probes without degeneracies and novel GI reagents. Compared to conventional multiplex RT-PCR assays, the monoplex and duplex TaqMan RT-PCR assays reported here are more rapid because they do not require additional nested amplification and/or additional confirmation steps, like hybridization or sequencing of the RT-PCR products. Identifying the possible source of an NoV outbreak related to shellfish consumption is crucial for early intervention and control, and we are currently investigating the possible options for developing TaqMan-based multiplex assays for rapid and reliable genotyping of NoV targeting region D of the capsid gene (33), as well as incorporating an internal control to address the presence of RT-PCR inhibitors. Although an RT-PCR-positive signal should be interpreted with caution since molecular methods cannot distinguish between infectious and inactivated viruses, recent inactivation studies with hepatitis A virus showed that

there was a clear correlation between the detection of a viral genome and the presence of infectious virus in a sample (3). In addition, by including assays for the detection of other enteric viruses, such as rotavirus and astrovirus, the multiplex detection of four different viruses in one reaction is a logical next step. The development of such TaqMan multiplex assays could have a tremendous impact on clinical diagnosis of enteric virus infections, and substantial time and costs could be saved if such an assay is coupled with automated nucleic acid extraction.

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